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Ethanol precipitation for purification of recombinant antibodies

Anne Tscheliessnig[a], Peter Satzer[a], Nikolaus Hammerschmidt[a,b], Henk Schulz[c], Bernhard Helk[c], Alois Jungbauer[a,b,*]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, A-1190 Vienna, Austria
[b] Austrian Centre of Industrial Biotechnology, Vienna, Muthgasse 18, 1190 Vienna, Austria
[c] Novartis Pharma AG, CH-4002 Basel, Switzerland

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ABSTRACT

Currently, the golden standard for the purification of recombinant humanized antibodies (rhAbs) from CHO cell culture is protein A chromatography. However, due to increasing rhAbs titers alternative methods have come into focus. A new strategy for purification of recombinant human antibodies from CHO cell culture supernatant based on cold ethanol precipitation (CEP) and $CaCl_2$ precipitation has been developed. This method is based on the cold ethanol precipitation, the process used for purification of antibodies and other components from blood plasma. We proof the applicability of the developed process for four different antibodies resulting in similar yield and purity as a protein A chromatography based process. This process can be further improved using an anion-exchange chromatography in flowthrough mode e.g. a monolith as last step so that residual host cell protein is reduced to a minimum. Beside the ethanol based process, our data also suggest that ethanol could be replaced with methanol or isopropanol. The process is suited for continuous operation.

1. Introduction

Purification of recombinant humanized antibodies (rhAbs) from cell culture supernatants is commonly performed by protein A chromatography followed by two to three additional chromatographic steps to obtain the purity required (Shukla et al., 2007; Sommerfeld and Strube, 2005). Depending on the rhAb the annual production volume is between 6 and 1000 kg/a with the pharmacy price value between 5000 and 400,000 US$/g (Hagel et al., 2007). In contrast, intravenous immunoglobulin (IVIG) preparations, which are usually used for the prophylactic prevention of infectious diseases in immunodeficient patients, have a significantly higher annual production volume, almost 100,000 kg/a at significantly lower cost, only ~91 US$/g (Hagel et al., 2007). The lower cost can be ascribed to the relatively simple process: The IVIG preparations are produced from collected human plasma using a series of cold ethanol precipitations (CEPs) known as the Cohn process (Buchacher and Iberer, 2006; Burnouf, 2007; Cohn et al., 1946, 1950; Moure et al., 2003; Radosevich and Burnouf, 2010). Since 1940s when this process has been first established by Cohn and coworkers, it has undergone several modifications to improve yield and purity (Buchacher and Iberer, 2006; Deutsch et al., 1946; Kistler and Nitschmann, 1962; Oncley et al., 1949). Still, the basic principle, variation of the five process parameters pH, ionic strength, ethanol concentration, protein concentration and temperature for selective precipitation of different plasma proteins, is still the same for every plasma fractionation process.

The simplicity and cost-effectiveness of the Cohn process make CEP an interesting option as an alternative, economic purification strategy for rhAbs from cell culture supernatant compared to other methods such as counter current loading (Godawat et al., 2012), counter current extraction (Rosa et al., 2013), or novel adsorption materials (Barroso et al., 2013; Borlido et al., 2013; Hilbrig and Freitag, 2012). However, the difference in the starting material (Tscheliessnig et al., 2013) makes the straight transfer of the Cohn process to the purification of rhAb from cell culture supernatant impossible: Despite the recent advances in rhAb titer in the last decade (Low et al., 2007; Shukla and Thoemmes, 2010) the concentrations of immunoglobulin (IgG) in human plasma are still higher (6–14 g/L, (Buchacher and Iberer, 2006)) than for rhAbs from cell culture supernatant (<5 g/L). Yet, due to the high complexity of the human plasma a large number of process steps are required to obtain the IVIG preparations in the required purity; this is at cost of yield (~30%) (Buchacher and Iberer, 2006). Also, the IgGs

* Corresponding author at: Department of Biotechnology, University of Applied Life Sciences, Vienna, Austria Muthgasse 18, A-1190 Vienna, Austria.
Tel.: +43 1476546226; fax: +43 1 3697615.
E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

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present in human plasma are highly heterogeneous, consisting of a range of different IgGs of various isoelectric points. In contrast the rhAbs present in the cell culture supernatant share the identical sequence thus are rather homogeneous compared to the IgGs present in human plasma (Kayser et al., 2011; Lingg et al., 2012). Only the different post-translational modifications observed result in the observed heterogeneity. This suggests that CEP could be a potential alternative for the purification of rhAbs from cell culture supernatant.

The advantage of CEP compared to other methods suggested to replace the conventional chromatography-based purification (Gagnon, 2012; Low et al., 2007) is, that besides its simplicity and cost-effectiveness, it also has a long safety record. IVIG preparations are produced using human plasma from a large pool of donors. This necessitates a tight control of potential contaminations with (known and unknown) viruses as well as prions (Buchacher and Iberer, 2006; Radosevich and Burnouf, 2010). It has been shown that the cold ethanol precipitation, also in combination with other orthogonal methods, is well capable to reach the required safety level (Buchacher and Iberer, 2006; Cai et al., 2002; Foster et al., 2000).

We present here the development of a purification platform for rhAb from CHO cell culture supernatant using CEP. In order to overcome co-precipitation of DNA with the rhAb during CEP we included $CaCl_2$ precipitation, which has been successfully established for clearance of not only DNA but also a number of protein impurities from the cell culture supernatant (Satzer et al., 2014). We established a platform based on CEP and $CaCl_2$ precipitation for the purification of four different rhAbs of different isoelectric point from CHO cell culture supernatant using a factorial design plan. The aim was a significant HCP reduction while maintaining yield. Aggregate formation is a permanent issue during CEP (Bull and Breese, 1978; van Oss, 1989; Yoshikawa et al., 2012), therefore the set-up of the CEP was designed to eliminate the detrimental effects of ethanol on proteins: good cooling of the suspension was provided and ethanol addition was slow to avoid precipitation due to excess heat caused by mixing of concentrated ethanol with an aqueous solution.

2. Materials and methods

All chemicals unless given otherwise were purchased from Merck (Darmstadt, Germany). All buffers for the analytical HPLC runs were prepared using HQ-H_2O, filtered through a 0.22 μm filter and degassed prior to use.

2.1. CHO culture supernatants

Clarified CHO culture supernatants of four rhAbs were provided by Novartis Pharma AG (Basel, Switzerland). They were stored at −20 °C for long-term storage or 4 °C for short-term storage. Prior to use the supernatants were filtered (0.22 μm).rhAb1 and rhAb2 are antibodies of the same amino acid sequence but expressed in different cell lines. Their isoelectric point was around 9.2. rhAb3 and rhAb4 were more acidic antibodies with a pI of 6.7 and 6.8 respectively. rhAb3 is prone to aggregation and was used to evaluate the effect that precipitation would have on such an antibody.

2.2. Solubility curves

The alcohols used were methanol (Methanol LCMS Chromasolv, Fluka), ethanol (Ethanol 96% Emprove exp) and isopropanol (Isorpopanol LiChrosolv). rhAb3 was used to evaluate if the alcohols differ in their behavior to cause aggregation.

Aliquots of 10 ml of the clarified cell culture supernatant of rhAb3 were transferred into the reactor vessels of an Integrity 10 (Thermo Fisher Scientific) and tempered to 4 °C. The respective alcohol was added over 1 h to a final concentration of 15.0% (v/v), 22.5% (v/v) or 30.0% (v/v) using a syringe pump (Ismatec, Wertheim-Mondfeld, Germany). Simultaneously the temperature was linearly decreased to either 0 °C or −5 °C. The precipitates were collected by depth filtration (GD/X, Whatman) and dissolved in 10 ml of histidine buffer (20 mM histidine, 100 mM NaCl, pH 6.0). All experiments were performed in triplicates.

Table 1
Factors and levels used for development of the purification strategies.

Factor	Levels	
	–	+
pH	6.5	8.5
Salt type	NaCl	$CaCl_2$
Conductivity	No salt added	To 40 mS/cm with respective salt
Temperature	9 °C	−10 °C
Ethanol concentration	30% (v/v)	40% (v/v)

2.3. Factorial design plan

The set-up and evaluation of the full factorial design plan was based on Montgomery (2009). The factors and levels selected are given in Table 1. The clarified culture supernatants of rhAb1, rhAb2 and rhAb3 were first adjusted to the required pH using 12.5% HCl or 1 M NaOH. If required, 5 M NaCl or 4 M $CaCl_2$ was added to a final conductivity of 40 mS/cm and the pH verified and amended if required. Any precipitate forming was not removed prior to CEP. The CEP was performed using the Integrity 10 (Thermo Fisher, Rochford, UK) which is an automated lab reactor with 10 reaction cells for which temperature and speed of a magnetic stirrer can be independently adjusted. 2 ml of the respective solution was cooled to 4 °C and then 96% (v/v) ethanol, also cooled to 4 °C, added to obtain a final concentration of 30% (v/v) or 40% (v/v) respectively. After 2 h of incubation at 300 rpm the precipitate of each cell was removed by filtration (0.22 μm) and the supernatant analyzed for DNA, IgG and total protein. Each set-up was evaluated in triplicates.

Using the effect estimates obtained a regression model was applied to calculate the solubility of the rhAbs, DNA and total protein within the limits of the factorial design plan applied. This allowed drafting different purification strategies before experimental evaluation. The levels of the factors given in Table 1 were selected to enable a regression model where a range of different conditions could be evaluated. For pH the levels were limited by the isoelectric points of the antibodies used and for ethanol the range was limited to the concentrations which were evaluated to result in a high precipitate fraction (see Fig. 1).

2.4. Lab-scale CEP

For the $CaCl_2$ precipitation the respective supernatant or solution was adjusted to pH 8.5 using 10 M NaOH. Then 5 M $CaCl_2$ was added to a final concentration of 250 mM $CaCl_2$. If required, the pH amended to pH 8.5 using 10 M NaOH. The precipitate was removed by centrifugation (4000 rcf, 15 min, RT). For the CEP supernatant obtained after $CaCl_2$ precipitation was adjusted to pH 6.5 using 25% HCl, then transferred to the EasyMax (Mettler Toledo, Gießen, Deutschland), an automated lab reactor and tempered to 4 °C. Using the integrated syringe pump a 96% (v/v) ethanol stock was added over 4 h to obtain a final concentration of 25% (v/v). Simultaneously the temperature was linearly decreased to −10 °C. The solution was mixed by the integrated over-head stirrer (350 rpm) and temperature and turbidity monitored by a temperature probe and IR probe respectively. After incubation for 2 h post ethanol addition the precipitate was collected by centrifugation (−10 °C, 4000 rcf, 15 min)

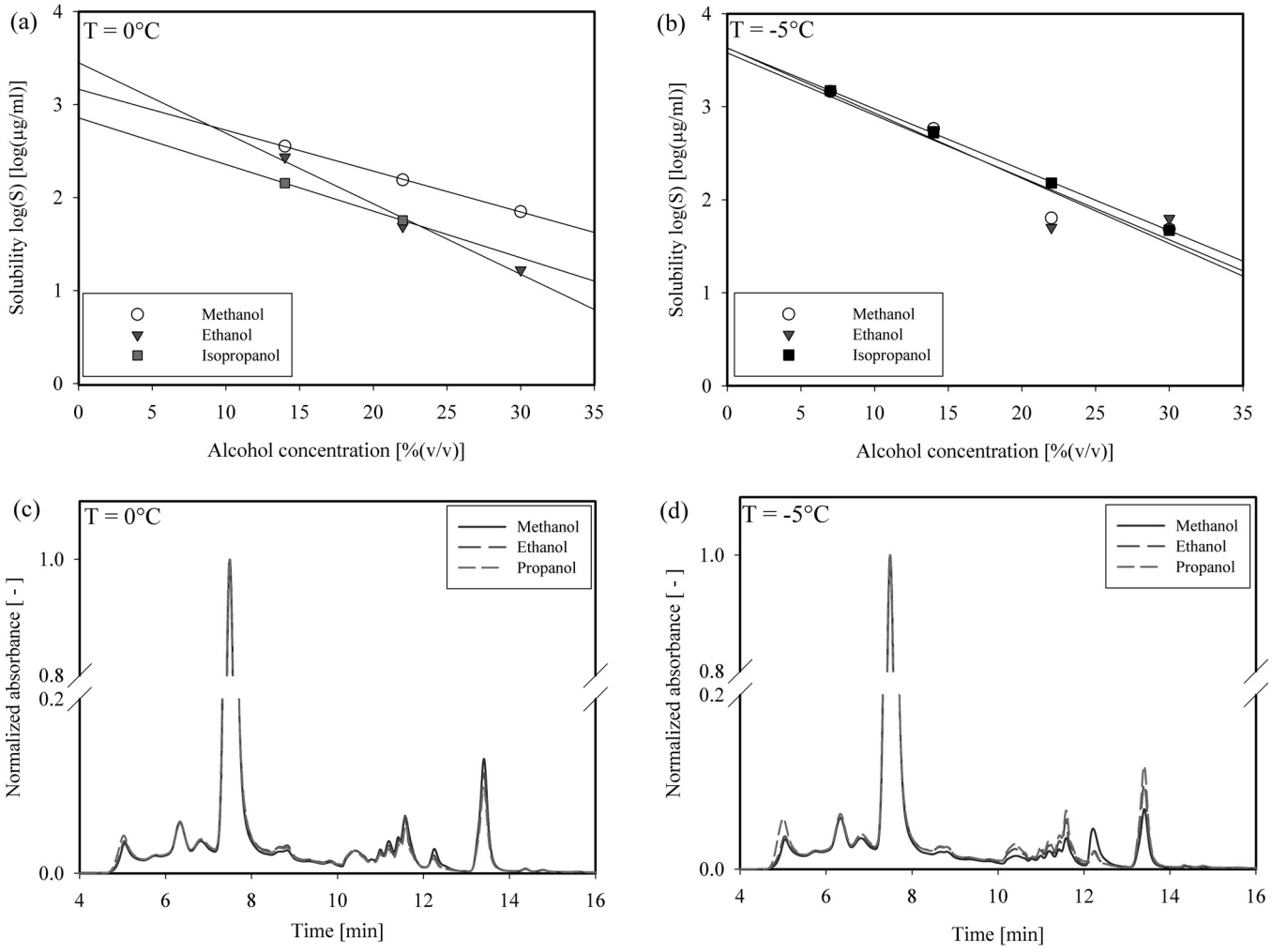


Fig. 1. Solubility curves at 0 °C and −5 °C show similar behavior for methanol, ethanol and isopropanol. Analytical SEC chromatograms of the respective dissolved precipitates are identical.

and then dissolved in 20 mM histidine, 100 mM NaCl, pH 6.5. All precipitations were performed in duplicates.

2.5. Initial screening for analytical anion-exchange chromatography

All experiments were performed using an Äkta Avant 25 at a flow rate of 4 ml/min and 10 ml/min respectively. The columns used were the CIM disk QA and the CIM disk DEAE (column volume 0.34 ml; Biaseparation, Ajdovscina, Slovenia) as well as a membrane adsorber, the Sartobind Q (volume: 0.41 ml, membrane area 15 cm^2 Sartorius, Goettingen, Germany). The column or membranes were equilibrated with 15 CV equilibration buffer (20 mM Tris, 25 mM NaCl, pH 8.5). The dissolved rhAb2 precipitate was present in dissolution buffer (20 mM histidine, 100 mM NaCl, pH 6.5). If required it was adjusted to pH 7.0 or 8.0 with 3 M Tris and/or diluted to a final

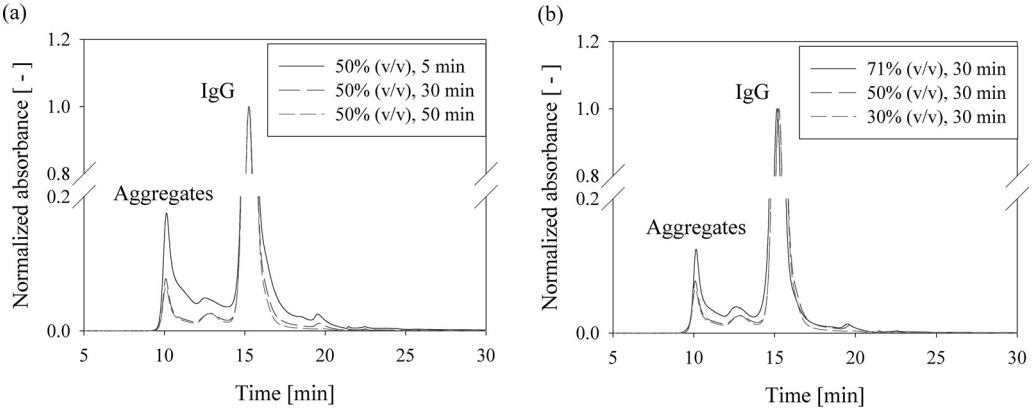


Fig. 2. Comparison of analytical SEC chromatogram of dissolved rhAb after precipitation with ethanol stocks of different concentration and different speed of ethanol addition.

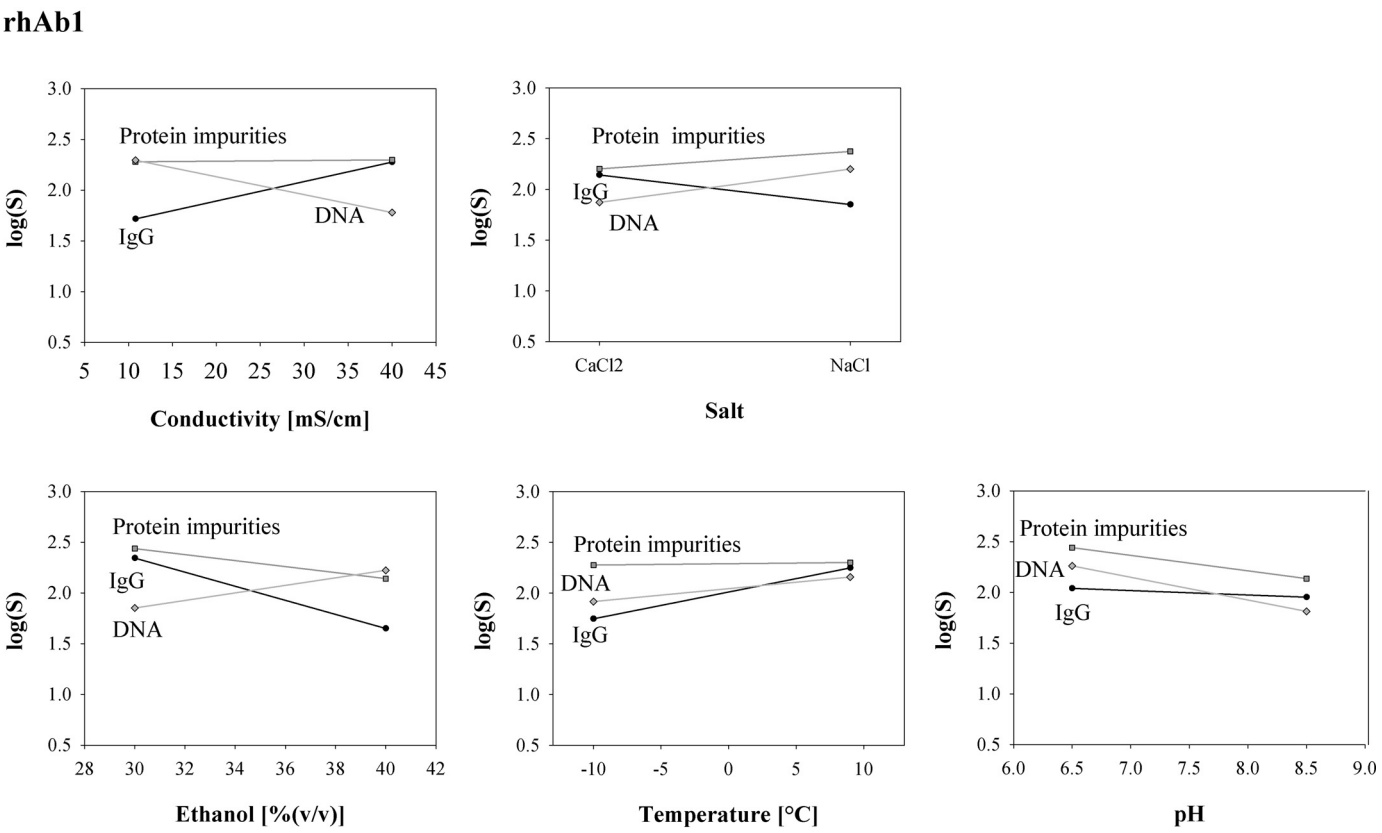


Fig. 3. Impact of the main factors on solubility of rhAb, DNA and protein impurities of the rhAb1 supernatant.

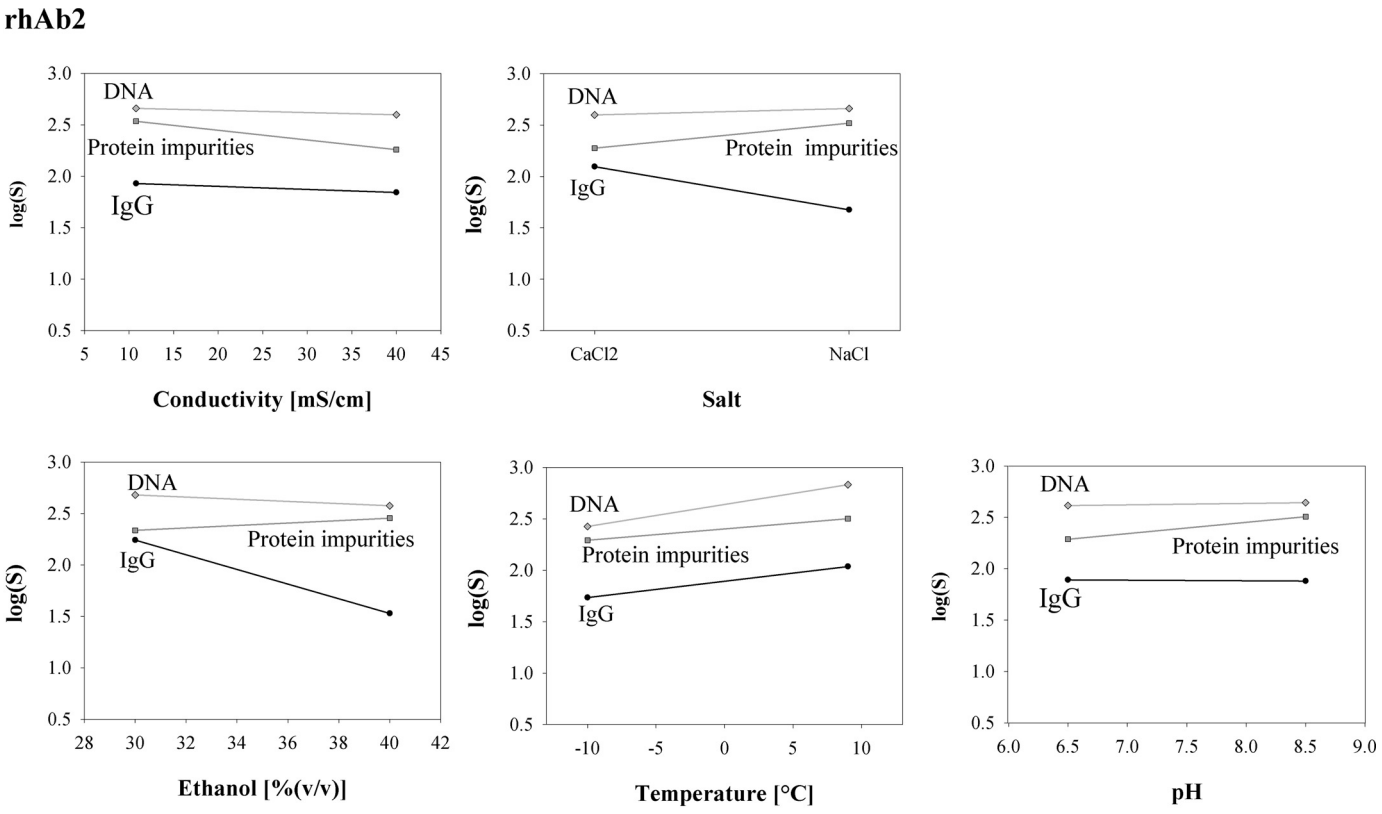


Fig. 4. Impact of the main factors on solubility of rhAb, DNA and protein impurities of the rhAb2 supernatant.

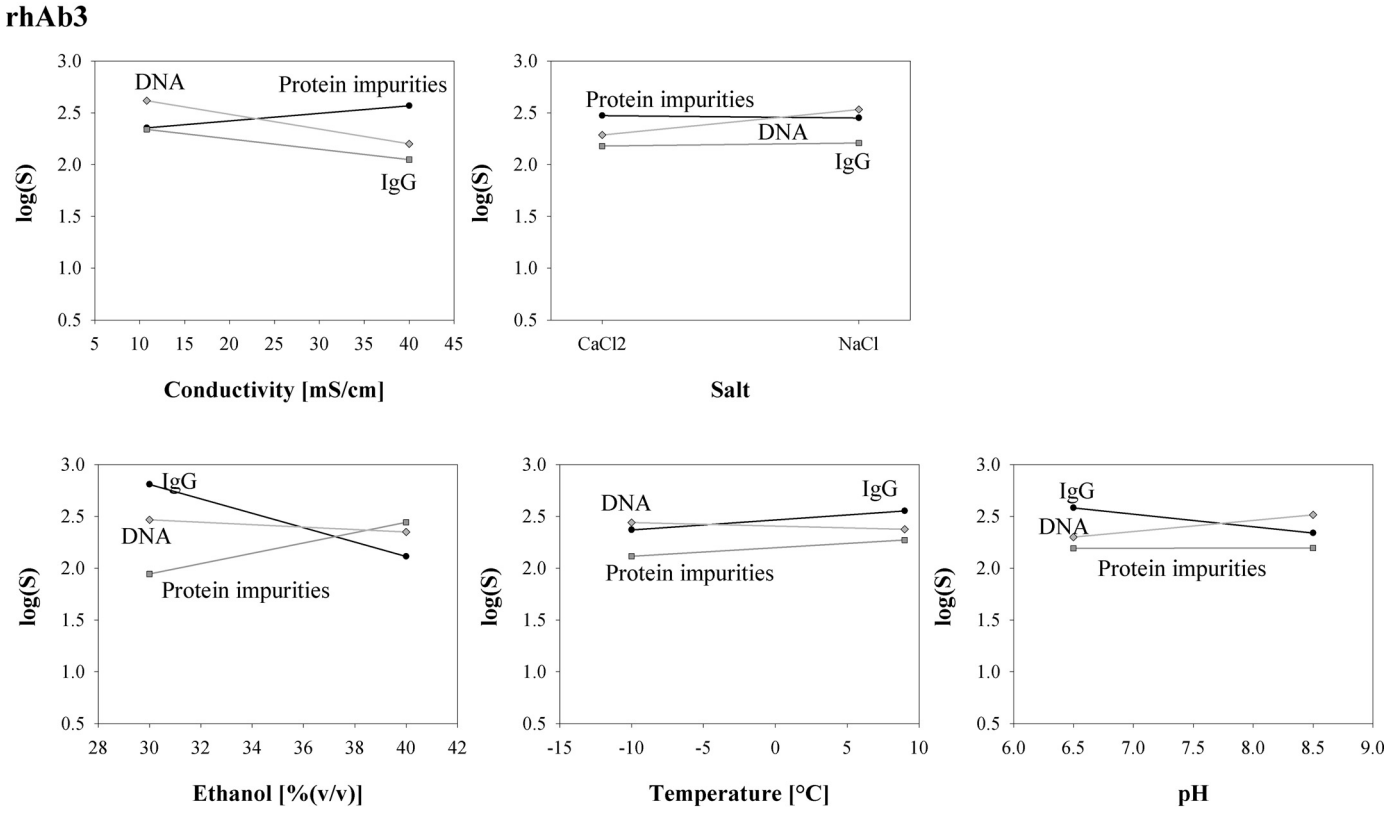


Fig. 5. Impact of the main factors on solubility of rhAb, DNA and protein impurities of the rhAb3 supernatant.

NaCl concentration 50 mM or 25 mM. The concentration of rhAb2 was 0.8 mg/ml except for buffers containing only 25 mM where the solution had to be diluted further (final: 0.4 mg/ml rhAb2). Bound contaminants were eluted using 20 mM Tris, 1 M NaCl, pH 8.0.

2.6. Lab scale CEP with subsequent analytical anion-exchange chromatography

The experiments were performed in triplicates. The CEP precipitations were performed as described previously, only the 2nd CEP precipitate was dissolved in 10 mM Tris, 25 mM NaCl, pH 8.0 instead of 20 mM histidine, 100 mM NaCl, pH 6.5. The dissolved precipitates were then filtered (0.2 μm, 25 mm GD/X PVDF syringe Filter, Whatman). The anion-exchange chromatography was performed using the Äkta Avant 25 at a flow rate of 4 ml/min. The CIM disk DEAE was equilibrated with 15 CV 10 mM Tris, 25 mM NaCl, pH 8.0, then the sample loaded and the flowtrough collected for analysis. The disk was regenerated using 20 mM Tris, 1 M NaCl, pH 8.0.

2.7. Analytical protein A chromatography

A method slightly modified as described by Tscheliessnig and Jungbauer (2009) was used. Briefly, a convective interactive media (CIM) monolithic disk with Staphylococcus Protein A (sProtein A) as ligand (CIM Protein A HLD, BIA Separations, Ljubljana, Slovenia) was connected to an Agilent 110 Series (Agilent Technologies Waldbronn, Germany). The disk was equilibrated using 30 mM sodium phosphate, 1000 mM NaCl, pH 7.4 (equilibration buffer) followed by injection of 25–100 μL of filtered (0.22 μm) sample. Unbound proteins were washed out by 0.5 ml of equilibration buffer and bound rhAb eluted with 2.0 ml 10 mM HCl (pH 2.0). The elution profile was monitored by measurement of absorbance at 280 nm and the concentration of rhAb was calculated by automated integration of the elution peak and comparison to a calibration curve prepared from a polyclonal IgG (Octagam, gift of Octapharma, Vienna, Austria).

2.8. Analytical SEC

If not mentioned otherwise SEC was performed using a Bio-SEC3 column (Agilent, Santa Clara, USA). The column and the respective guard column were connected to an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany). The column was equilibrated (0.2 M sodium phosphate, 0.1 M potassium sulfate, pH 6.0) at a flow rate of 1.2 ml/min. 100 μl of filtered (0.2 μm, Millipore, Carrigtwohill, Ireland) sample was injected and the UV absorbance monitored at 280 nm. Aggregates and high-molecular weight impurities are defined as peaks observed at an elution time lower than 7 min and remaining impurities are defined as peaks observed at an elution time higher than 9 min. For rhAb4 analytical SEC was performed using the TSK G3000 SWxl column (Tosoh Bioscience, Tokio, Japan). The column and the respective guard column were connected to an Agilent 1100 Series (Agilent Technologies) and tempered to 30 °C. The column was equilibrated (150 mM potassium phosphate, pH 6.5) at 0.4 ml/min and 10 μl of filtered (0.2 μm, Millipore) sample was injected. The UV absorbance was monitored at 210 nm. Aggregates and high-molecular weight impurities are defined as peaks found at an elution time lower than 20 min and remaining impurities are defined as peaks observed at an elution time higher than 25 min.

2.9. DNA quantification

DNA was quantified using the Quant-iT PicoGreen DNA kit (Invitrogen, Paisley, UK) in microplate format according to the manufacturer's instructions.

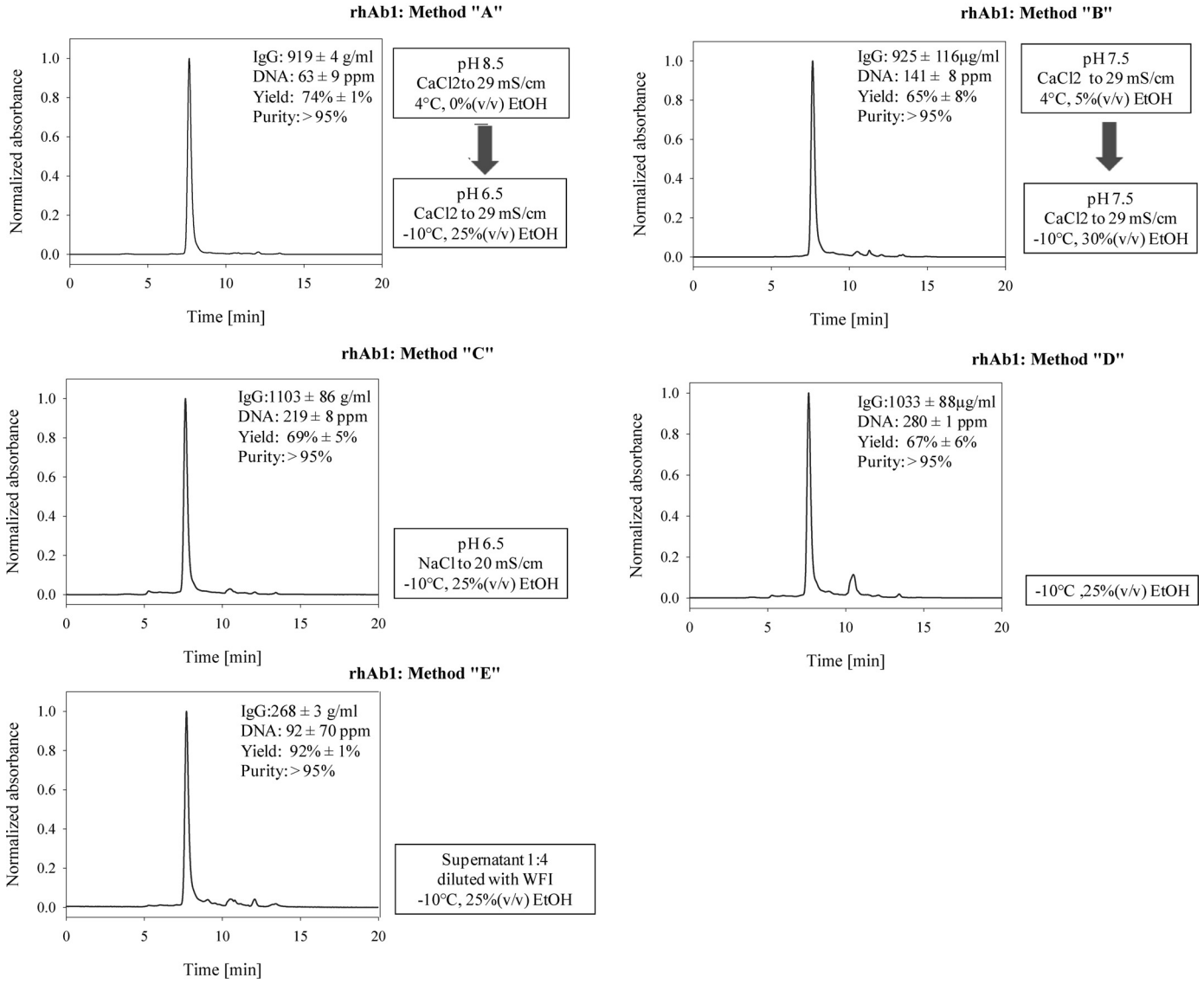


Fig. 6. Different precipitation methods evaluated for rhAb1 supernatant. Method "D" and "E" are for comparison: method "D" is without modification of the supernatant; method "E" is using 1:4 diluted (with WFI) supernatant.

2.10. HCP ELISA

First a microtiter plate (Immuno 96 MicroWell Plates, MaxiSorb, NUNC, Roskilde, Denmark) was coated with goat anti-CHO HCP antibody (3G-0016-AF, Cygnus, Southport, NC, USA) diluted 1:400 in 200 mM sodium carbonate, pH 9.4 overnight at 4 °C. The plates were then blocked with 3% bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, USA) in TBS/Tween (500 mM Tris, 1500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4) for 1 h at 37 °C. For each plate a duplicate standard curve (1.6–200.0 ng/ml) was prepared by serial dilution (1:2) of a CHO HCP stock (F553H, Cygnus) in 1% BSA in TBS/Tween. The samples were diluted likewise and after transferring samples and standards to the microtiter pate, it was incubated for 1 h at 37 °C. After incubation with the goat anti-CHO HCP HRP concentrate (3G-0016-AF, Cygnus) diluted 1:2000 in TBS/Tween for 1 h at 37 °C the TMB Peroxidase EIA Substrate Kit (Bio-Rad, Hercules, USA) was used as recommended by the manufacturer for staining. The enzymatic reaction was stopped by addition of 50 µl of 1 N H_2SO_4 to each well. The absorbance at 405 nm was measured using a Tecan Infinite F500 plate reader (Tecan, Salzburg, Austria).

3. Results

3.1. Alternative organic solvents

Ethanol is a common precipitant for proteins (Gemili et al., 2007; Golunski et al., 2011; Wilcockson, 1975) as well as DNA (Wilcockson, 1975). Its frequently cited advantages are (i) its miscibility with water, (ii) no formation of explosive gaseous mixtures under normal working conditions, (iii) it is evaporable and (iv) chemically inert, has a (v) low toxicity, results in the (vi) reduction of freezing point and (vii) it is cheap and easily available. We also evaluated other alcohols for their applicability to precipitate IgG from a clarified CHO cell culture supernatant. We found that methanol and isopropanol have similar efficiency for precipitation (see Fig. 1). However, methanol is toxic and its application of the purification of biopharmaceutical proteins should be considered with care. We expect a similar behavior for 1-propanol as for isopropanol. Starting with butanol the miscibility of the alcohols was too low to be used for precipitation.

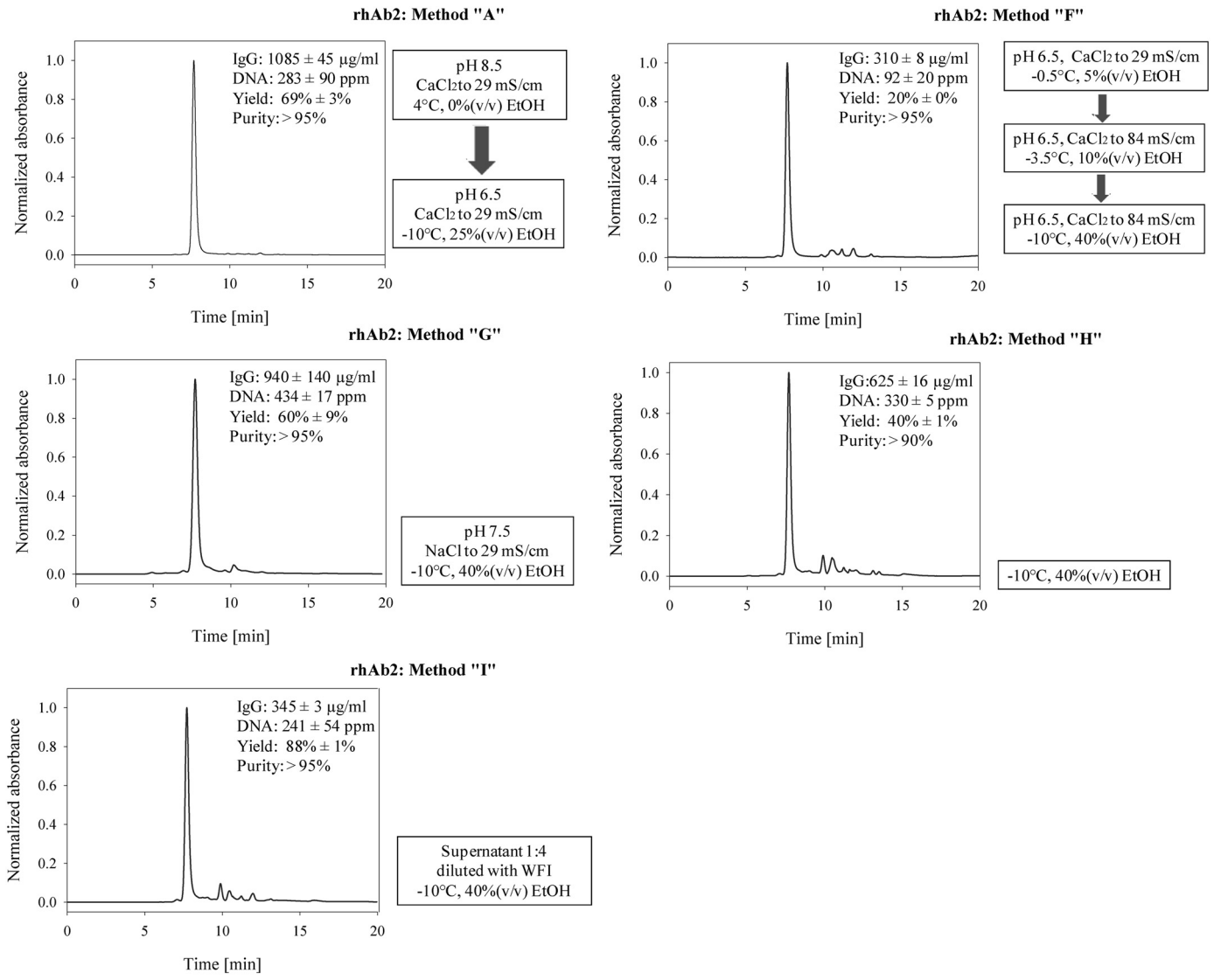


Fig. 7. Different precipitation methods evaluated for rhAb2 supernatant. Method "H" and "I" are for comparison: method "H" is without modification of the supernatant; method "I" is using 1:4 diluted (with WFI) supernatant.

3.2. Development of cold ethanol precipitation (CEP)

Based on the Cohn process we selected pH, ethanol concentration and temperature for the factorial design plan. Additionally, instead of ionic strength we also included conductivity as factor and adjusted it using either sodium chloride or calcium chloride. We found that it was important to add ethanol at a slow flow rate to avoid formation of aggregates (see Fig. 2). Addition of ethanol over 4 hours ensured that no or only few aggregates formed when adding 96% (v/v) ethanol.

All experiments were performed in triplicates using the supernatant of rhAb1–rhAb3. The solubility of IgG, DNA and protein impurities under the selected conditions was evaluated by determining their concentration in the supernatant obtained after precipitation. The impact of the main factors on the solubility of DNA, IgG and protein impurities for rhAb1 is found in Fig. 3. For rhAb2 and rhAb3 the impact of the main factors is found in Figs. 4 and 5, respectively. Similar, the trends of the interactions of the main factors can be evaluated by plotting the respective data (figure not shown). For all rhAbs we find that the main factors ethanol concentration and temperature have a significant influence on solubility of the respective rhAb. Also we find that interaction of the factor conductivity and temperature affects solubility for all rhAbs: for higher conductivity a lower temperature is required to ensure low rhAb solubility. For other main factors or interacting factors we find no similarity between all rhAbs. From the data it is however clear, that pH has only little influence on rhAb solubility during CEP. Regarding solubility of DNA we found for all rhAbs that solubility increased with increasing temperature; for all other factors and interactions the effect depends on the supernatant used. In case of the supernatants of rhAb1 and rhAb2 we find that the types of salt as well as the ethanol concentration have significant impact. Regarding protein impurities the factors having a significant impact on solubility are dependent on the rhAb supernatant evaluated. For the main factors we find only two factors which give the same trend for all mAb supernatants: with increasing salt and increasing temperature the solubility of the protein impurities increases. For the interacting factors no agreement can be found at all.

In order to obtain a first estimation of the possible purification conditions we used the data obtained from the factorial design plan to apply a regression model. We obtained a set of functions describing the solubility of the respective rhAb, DNA and protein impurity for each supernatant. We then calculated the effect of different purification conditions and drafted different purifications.

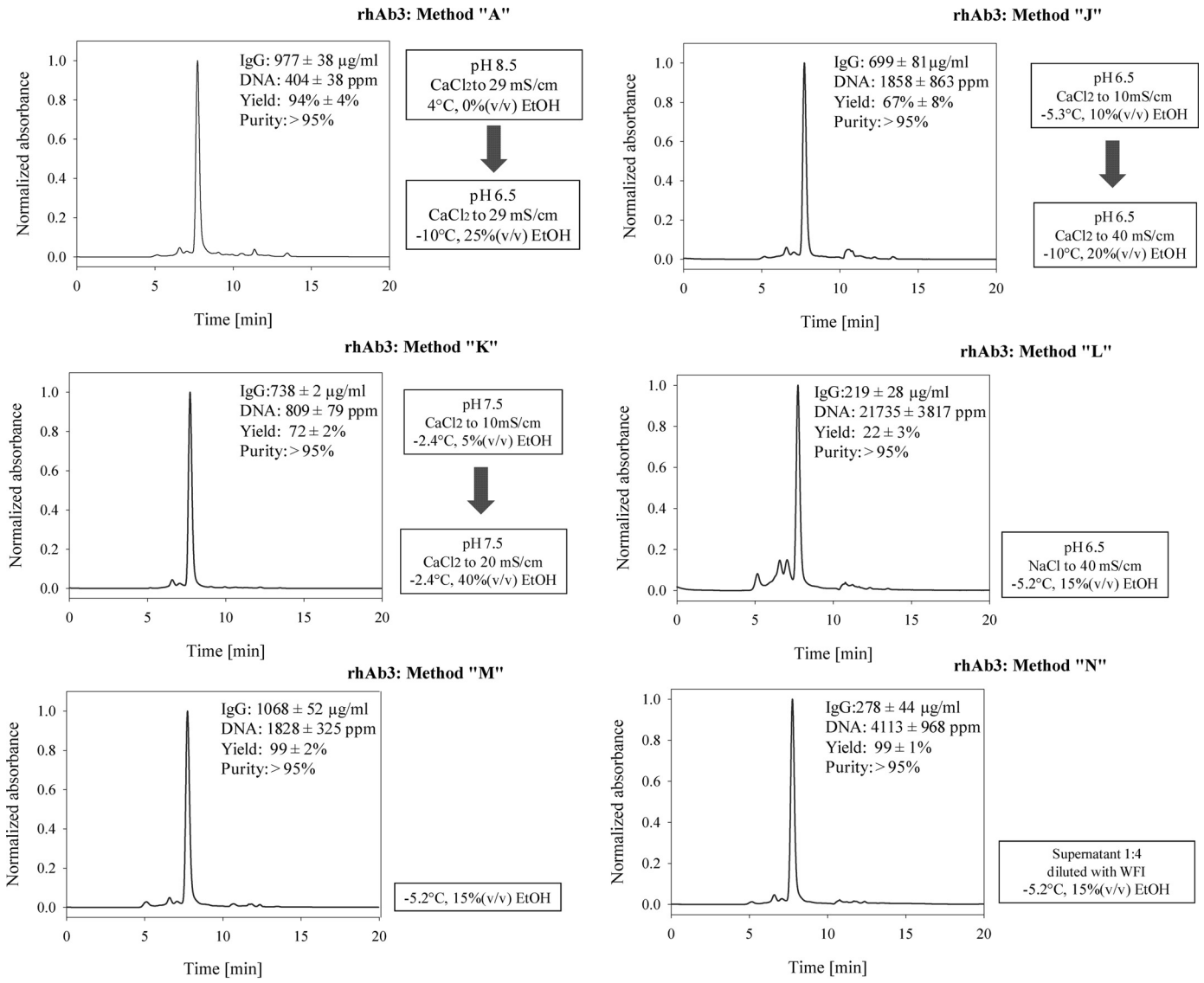


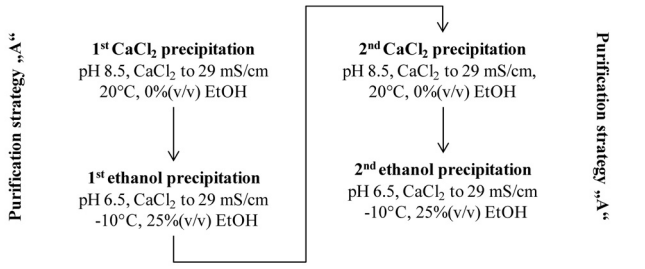
Fig. 8. Different precipitation methods evaluated for rhAb3 supernatant. Method "L" and "M" are for comparison: method "L" is without modification of the supernatant; method "M" is using 1:4 diluted (with WFI) supernatant.

The aim of each precipitation step was to selectively precipitate either the rhAb or DNA and total protein. As comparison we also always evaluated ethanol addition to the supernatant without prior adjustment of conductivity or pH.

We then evaluated different purification strategies (see Figs. 6–8) and found that one purification strategy, labeled "A" could be used for all antibodies tested. As given in the table in Fig. 9 we also found for rhAb2 that the repeated execution of the purification strategy "A" resulted in a 155× reduction of CHO HCP. Hence we continued to use the repeated execution of purification strategy "A" for CEP.

3.3. Lab-scale purification

We performed the CEP purification also at larger lab-scale (70 ml) to verify increase of yield upon up-scaling. With up-scaling also minor adjustments of the method were performed: (i) instead of addition of $CaCl_2$ to a specific conductivity (29 mS/cm) we estimated the appropriate concentration of $CaCl_2$ (250 mM) required to reach this conductivity. This simplified precipitation at large-scale. (ii) We performed the $CaCl_2$ precipitation at room temperature. In



	IgG [µg/ml]	HCP (ppm)	DNA (ppm)	Purity	Yield (step/overall)	HCP reduction
Supernatant	1383	44792	752	12%		
1st $CaCl_2$ precipitation	1020	n.d.	n.d.	14%	75%/75%	
1st CEP	680	4505	83	>90%	77%/58%	10
2nd $CaCl_2$ precipiation	531	n.d.	n.d.	>90%	71%/41%	
2nd CEP	250	289	149	>95%	64%/26%	155

Fig. 9. Set-up of purification strategy "A" when executed repeatedly. Mass balance for repeated execution of purification strategy "A".

Table 2
Yield, IgG monomer content, purity and HCP concentration of the large lab-scale purifications (70 ml) of rhAb1, rhAb2, rhAb3 and rhAb4. Each precipitation was performed as duplicate.

	IgG [μg/ml]	Yield step/overall	IgG monomer	Purity	DNA [ppm]	HCP [ppm]	HCP reduction step/overall
rhAb1							
Supernatant	1222				1021	54,212	
1st $CaCl_2$	1029	94%/94%				44,641	1.2/1.3
1st CEP	874	85%/80%			628	6922	6.5/7.8
2nd $CaCl_2$	684	86%/69%				3157	2.3/17.2
2nd CEP	636	93%/64%	>99.9%	>99.9%	557	650	4.9/83.4
rhAb2							
Supernatant	2563.4				3037	109,231	
1st $CaCl_2$	2379.4	98%/98%				26,773	4.1/4.1
1st CEP	2172.4	91.3%/89%			1294	15,224	1.8/7.4
2nd $CaCl_2$	1991.7	98.1%/88%				3865	3.9/28.9
2nd CEP	1816.4	91.2%/80%	99.90%	99.90%	350	1202	3.2/92.1
rhAb3							
Supernatant	1953	0	0		2659	180,099	
1st $CaCl_2$	1808	99%/99%				n.d.	
1st CEP	1649	89%/88%			1336	n.d.	
2nd $CaCl_2$	1487	>99%/88%				n.d.	
2nd CEP	1391	94%/83%	89.50%	89.50%	545	8276	9.37/20.0
rhAb4							
Supernatant	3322				358	81,752	
1st $CaCl_2$	2826	92%/92%				n.d.	
1st CEP	n.a.	n.a.			n.d.	n.d.	
2nd $CaCl_2$	2337	89%/82%				n.d.	
2nd CEP	2162	93%/76%	99.40%	99.40%	80	3702	22.09/48.65

previous experiments we verified that change of temperature had no effect on the yield or purification efficiency of the $CaCl_2$ precipitation (data not shown). (iii) No washing step was used for the CEP precipitate. Besides for the previously used rhAbs (rhAb1–rhAb3) the method was also applied for rhAb4.

We monitored the CEP step using an IR probe to measure the increasing turbidity. We found that the precipitation is a fast, step-like process starting at around 17% (v/v) ethanol and −6 °C (see Fig. 10).

Fig. 11 gives the analytical SEC and the results of the final product for the purification of rhAb1–rhAb4. A detailed mass balance can be found in the Table 2. For rhAb1 an overall yield of 64% was achieved, this could be due to the low IgG concentration in the supernatant (~1 g/L) as compared to the other rhAb supernatants. As around 0.1 g/L stay in solution under the selected conditions (data not shown), we can only expect about 90% overall yield during each CEP step. This is in agreement with the data found in Table 2. Additional losses during the $CaCl_2$ precipitation of the impurities

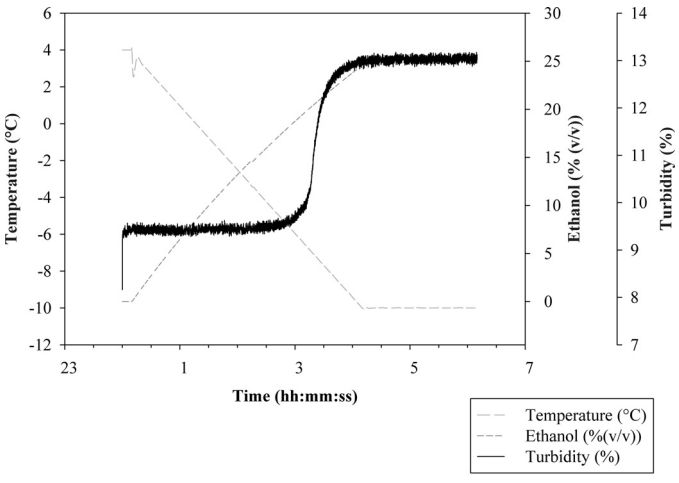


Fig. 10. Temperture profile, IR probe response and ethanol concentration of a typical CEP step at large lab-scale (70 ml).

then result in the low overall yield. We find that the purified rhAb1 (2nd CEP precipitate) is of high purity (>99.9%) and contains no aggregates. This can also be seen in the chromatogram of the analytical SEC (Fig. 11). The final HCP concentration of 650 ppm results in an overall HCP reduction of ~80×. As can be seen for rhAb1 the CEP step is more efficient in HCP removal than the $CaCl_2$ precipitation step. However, we found that the $CaCl_2$ precipitation step is required for an efficient removal of the HCPs (data not shown).

For rhAb2 a higher overall yield was observed (80%) which is not unexpected considering the higher starting concentration (see Table 2). Also the purified rhAb2 has no aggregates and is of high purity (99.9%). This is also confirmed by analytical SEC (Fig. 11). The HCP concentration is higher than for rhAb1 but due to the higher HCP content in the supernatant of rhAb2 a slightly higher HCP reduction (>90×) is achieved.

For rhAb3 yield is highest among all rhAb supernatants tested (>83%). However, rhAb3 is prone to aggregation and as can be seen in Fig. 11 or also in Table 2 a significant amount of aggregates can be found. HCP removal is low, only 20×.

For rhAb4 the purification strategy has not been tested prior to upscale. However we found that it worked well (Table 2), resulting in an overall yield of 76% a high purity (>99%) and, as also seen in Fig. 11, a low aggregate content. We found that HCP reduction was relatively low (<50×). This could not be further improved by modification of the $CaCl_2$ precipitation or CEP (data not shown).

3.4. Comparison to protein A chromatography

Comparing our purification based on precipitation with a protein A-based purification performed in-house we find better HCP clearance (pA: 5232 ppm; CEP: 650 ppm) and aggregate concentration (pA: <1.4%; CEP: <0.1%) for rhAb1 (Satzer et al., 2014). However, the protein A purification is not optimized and better HCP and aggregate clearance could be expected. Follman et al. (Silva et al., 2012) obtained a reduction to 300 ppm equivalent to a ~70× reduction. Yield is in any case higher for the protein A-based purification (84%), which is obvious as for the affinity-based purification only a single purification step is required, while for the precipitation-based purification four steps are required. However, we found an

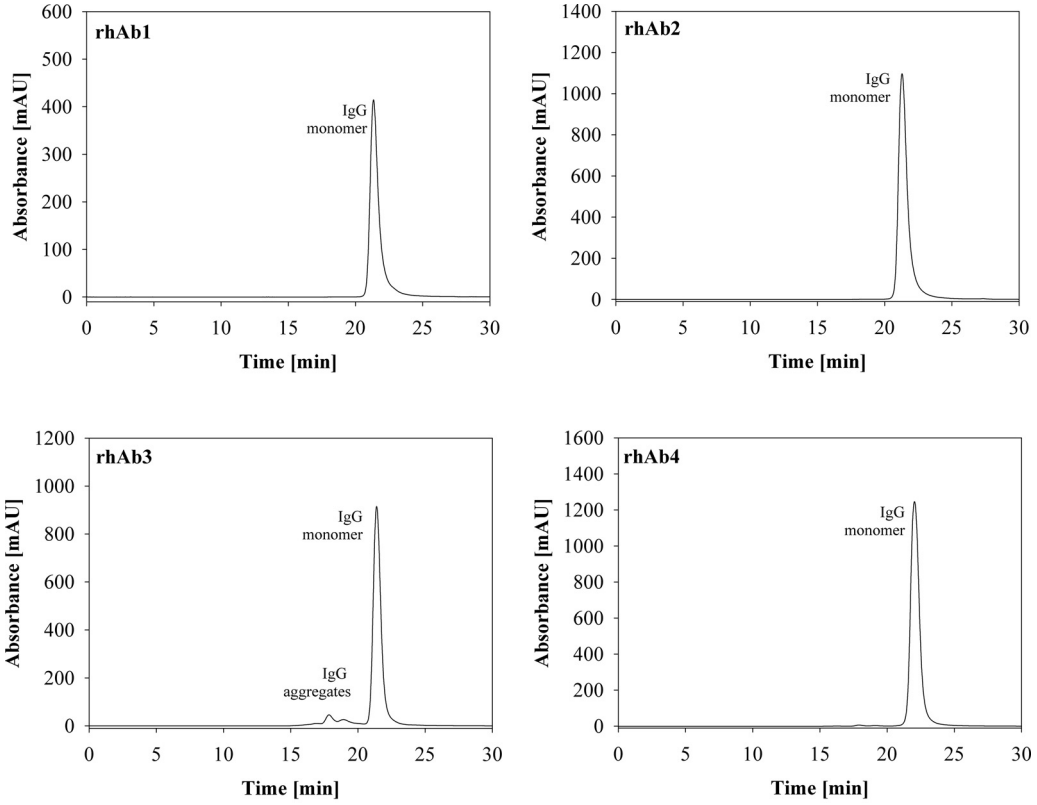


Fig. 11. Analytical SEC chromatograms for purified rhAb1, rhAb2, rhAb3 and rhAb4 from large lab-scale (70 ml) experiment.

increase in yield from 26% at small lab-scale (10 ml) to 64% at large lab-scale (70 ml) and we expect further improvement upon further up-scaling.

3.5. Anion-exchange chromatography (AEX)

We screened the conditions for AEX of rhAb2 with regards to pH (6.5, 7.0 and 8.0), NaCl concentration (25 mM, 50 mM or 100 mM) and column (CIM disk DEAE, CIM disk QA and Sartobrand DEAE). Highest HCP removal was found for pH 8.0 and 25 mM NaCl using the CIM disk DEAE (see Fig. 12). We performed a larger lab-scale precipitation (70 ml) including the developed AEX as final step. For second CEP, previous to the AEX step the precipitate was dissolved in the buffer required for AEX (10 mM Tris, 25 mM NaCl, pH 8.0). Details of the purification are found in Fig. 13. We obtained a yield of 78% with a low DNA (121 ppm) and HCP (80 ppm) content. Fig. 13 also gives the SEC chromatogram (TSK G3000 SWxl); a second peak can be found subsequent to the IgG peak which is caused by the sample buffer.

4. Discussion

We have developed a purification process for rhAbs from clarified CHO culture supernatant using $CaCl_2$ precipitation and cold ethanol precipitation (CEP). We showed that the process can be applied to rhAbs of different isoelectric point. HCP clearance was in some cases very efficient (80–90×). A more detailed characterization of the HCPs present in the different supernatants might be helpful in identifying the difference in the HCP profile of the different supernatants. Yield was around 60–90% and we expect it to increase further upon further upscaling. The yield is also dependent on the initial concentration of antibodies in the supernatant. For precipitation the residual concentration of antibody in the supernatant is defined by the precipitation conditions, applied, e.g. pH, temperature, salt concentration, precipitant. This concentration is constant regardless of the initial antibody concentration. Now, if the initial antibody concentration is high, then this residual non-precipitated antibody is a smaller fraction than for lower initial concentrations. Therefore yield will increase with the initial concentration.

Ethanol could also be replaced by methanol or isopropanol if desired. The method is suited for a continuous operation, which would further improve the economy of such a process

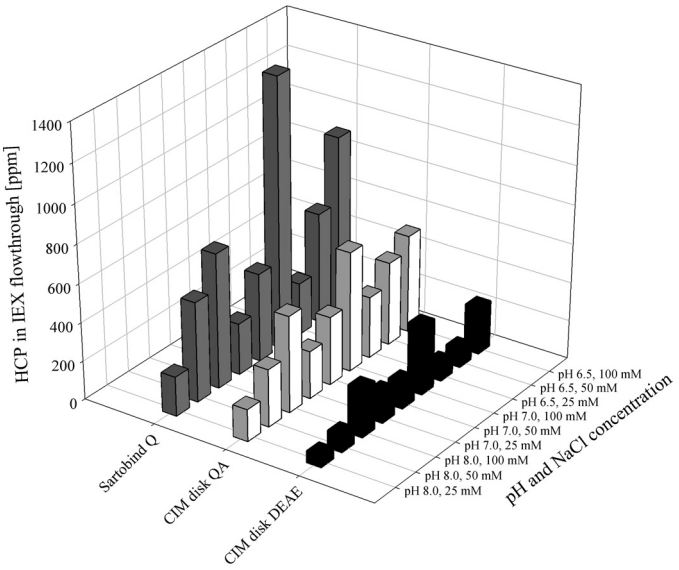
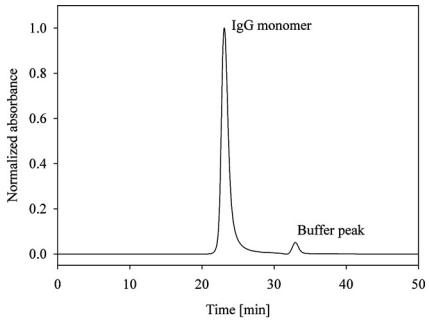


Fig. 12. HCP concentration in flowthrough as measured in the screening (pH and NaCl) of the CIM disk QA, CIM disk DEAE and Sartobind Q.



rhAb2	IgG	Yield		IgG monomer	DNA	HCP	HCP Reduction	
	[µg/ml]	step	overall		[ppm]	[ppm]	step	overall
Supernatant	2509 ± 0				2583 ± 0	136424 ± 0		
1st $CaCl_2$	2272 ± 28	96% ± 1%	96% ± 1%		30 ± 1	107010 ± 3387	1.3 ± 0.0	1.3 ± 0.0
1st CEP	2161 ± 41	95% ± 2%	91% ± 2%		166 ± 58	28350 ± 2559	3.8 ± 0.4	4.8 ± 0.5
2nd $CaCl_2$	1845 ± 27	91% ± 2%	83% ± 1%		<LLOQ	6406 ± 801	4.5 ± 0.5	21.5 ± 2.5
2nd CEP	1743 ± 3	96% ± 1%	79% ± 2%	99.92% ± 0.02%	136 ± 37	1254 ± 182	5.1 ± 0.3	110.3 ± 15.4
DEAE AEX	1715 ± 49	99% ± 1%	78% ± 2%	99.95% ± 0.01%	121 ± 21	80 ± 14	15.7 ± 1.1	1739.2 ± 326.7

Fig. 13. Chromatogram of analytical SEC of final product (DEAE AEX flowthrough) and mass balance for purification of rhAb2.

(Jungbauer, 2013). In principle the method is also suited for separation of antibodies produced with other host systems such as PerC.6 (Kuczewski et al., 2011) or even microbial cells such as *Pichia pastoris* (Maccani et al., 2014).

Additional the CEP for immunoglobulins from human blood plasma had been developed as a continuous process: In order to avoid the Cohn fractionation being carried out batch-wise in vessels of 2000–10,000 L a fractionation process based on continuous small volume mixing (CSVM) was developed and implemented in the fractionation center of the Scottish National Blood Transfusion Service which was commissioned in 1975 (Foster et al., 1986). A truly continuous operation (24 h/day) was also tested over 6 days and had a capacity of 338,000 L a year. The potential of a continuous precipitation reactor was therefore clearly demonstrated (Foster et al., 1986). For our process an additional $CaCl_2$ precipitation would need to be implemented. The last step of our purification method, AEX, is operated in flowthrough mode, which would be easily implemented in a continuous process. We found our process to be comparable to our in-house protein A purification suggesting that the $CaCl_2$/CEP purification method could be used to replace the initial capture step of conventional rhAb purification processes. For rhAb2 we have also implemented a final chromatographic anion-exchange chromatography step for additional CHO HCP removal. With this step the CHO HCP could be decreased to as low as 80 ppm compared to ~1200–1500 ppm for $CaCl_2$ precipitation/CEP alone. This data was obtained under laboratory conditions, applying the conditions required during commercial production (e.g. sanitized equipment, WFI, . . .) a higher purity could be expected. In production environment validated cleaning procedures minimize risk of carry over. With increasing size we also expect reduced carry over, because the surface to volume ratio shrinks. The purity was higher with antibody with high pI. This is explained by the nature of CHO HCP. A lot of these proteins are in the acidic range and discrimination due to pH is more difficult.

The method introduced has been established for four different antibodies. While we found that the method as described worked well for the antibodies selected, it is understood, that adaptation of this method may be required to make it suitable for other antibodies. With the capabilities provided by automated platforms (Berg et al., 2014; Nfor et al., 2012; Silva et al., 2012; Treier et al., 2012a, 2012b) such a screening process could be easily set up and provide the tools for fast process development. We also tested PEG precipitation and combination of CEP and PEG for rhAB purification. Also these methods result in high purity and yield and a platform approach could be used for optimization. We see a big potential in combing these methods.

5. Conclusion

We have shown the applicability of the cold ethanol precipitation for the purification of recombinant antibodies from cell culture supernatant. The advantage of this process is that the $CaCl_2$/CEP purification method requires only simple equipment such as stirred tanks and centrifuges, making it an easily scalable process. Also, CEP is already an established method in the biopharmaceutical industry, being the standard method of plasma fractionation. The lower costs of the IVIG preparation prepared by the Cohn process as compared to conventional rhAb preparations from CHO supernatants suggest that the $CaCl_2$ precipitation/CEP could be an improvement in the production of rhAbs.

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